Analysis of the Role of MLL-PTD in Hematopoeisis

Honors Research Thesis
Presented in partial fulfillment of the requirements for graduation with *Honors Research Distinction in Biology* in the College of Arts and Sciences at The Ohio State University

Daniel Adam Yanes

The Ohio State University

9/24/2012

Abstract

Acute myeloid leukemia (AML) is a cancer with a harsh treatment regimen, affecting primarily the elderly. The partial tandem duplication mutation of the mixed lineage leukemia gene (*MLL*-PTD) is seen in 5-10% of patients with AML and is associated with a poor prognosis and a high chance of relapse. Molecular studies demonstrate that MLL-wild type (WT) transcripts are not expressed in *MLL*-PTD+ AML patient blasts. It is thus important to study how the PTD mutation functions in the absence of *MLL*-WT. We first generated a mouse model that carries the *Mll*-PTD mutation on one allele while the second *Mll* gene remained normal [wildtype (WT)]. The generation of the homozygous *Mll*-PTD animals and also hemizygous *Mll*-PTD mice (lacking the *Mll*-WT gene) was lethal in both cases. This demonstrated that the Mll-WT is required for full development. Thus, to obtain a scenario that would mimic that observed in humans with *MLL*-PTD (absence of *MLL*-WT expression), we developed a new mouse model that allows for the removal (knockout) of *Mll*-WT while retaining the *Mll*-PTD after the newborn heterozygous pups obtained adulthood (conditional knockout approach). *Mll*-PTD heterozygous mice were crossed to *Mll*-conditional knockout animals to generate $Mll^{PTD/F}$ animals also expressing *Mx1*-driven Cre recombinase. Following induction of Cre recombinase with poly I:C, exons 3 and 4 of the floxed *Mll* (*Mll*-F) are removed, creating *Mll*-ΔN, which lacks the subnuclear localization domain needed for normal nuclear function. The resulting PTD/ΔN animals thus maintain *Mll*-PTD as the only nuclear functional copy of *Mll* in hematopoietic cells. PCR analysis of bone marrow and spleen from animals with conditionally induced Cre expression demonstrated complete deletion of the *Mll*-F allele. $Mll^{PTD/\Delta N}$ animals survived past 500 days ($p<0.001$) with no signs of bone marrow failure, while $Mll^{\Delta N/\Delta N}$ animals died of bone marrow failure 18 days after induction on average. Histopathological analysis of BM samples

revealed marked hypocellularity in $Mll^{\Delta N/\Delta N}$ animals, but no signs of hypocellularity in $Mll^{PTD/\Delta N}$ animals or age-matched normal WT controls. $Mll^{PTD/\Delta N}$ animals demonstrated no differences in white blood cell count (p=0.1693) or hematocrit (p=0.0916) upon sacrifice compared to non-moribund controls. Preliminary qRT-PCR analysis of BM and spleen samples at 12 days post-induction revealed a similar 4-5 fold increase of the *Mll* transcriptional target *HoxA9* in $Mll^{PTD/\Delta N}$ and $Mll^{PTD/F}$ animals relative to $Mll^{F/F}$ animals, consistent with the hypothesis that *Mll*-PTD acts in a gain-of-function manner even in the absence of *Mll*-WT. $Mll^{PTD/\Delta N}$ mice show no signs of developing acute leukemia suggesting the absence of the WT allele in pts with MLL-PTD AML, may be a consequence rather than a cause of leukemic transformation. However, further analysis of this unique mouse model provides evidence that the *Mll*-PTD mutation is capable of sustaining normal levels of hematopoiesis in the absence of any *Mll*-WT expression. With a further understanding of the biology of *Mll*-PTD positive AML, *Mll*-PTD itself or its downstream targets can be targeted to improve the prognosis of those afflicted with this disease.

Introduction:

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a cancer of myeloid cells, which are any blood cells that are not lymphocytes. In AML, rapid proliferation of myeloid progenitors known as myeloblasts, and a decrease in ability to differentiate and undergo erythropoiesis, thrombopoeisis, or leukopoiesis leads to accumulation of immature cells in the bone marrow and blood. AML is primarily a disease of the elderly, with a median age in the United States of 68 years, and incidence increases nearly tenfold for people over the age of 65.[2] Older patients have a poorer prognosis than younger patients due to differences in the pathology and basis of the

disease, as well as their ability to tolerate the current treatments of AML. Current treatment regimens for AML include cytotoxic chemotherapy, bone marrow transplantation, or a combination of the two. Chemotherapy regimens often use an intense treatment of drugs such as cytarabine, daunorubicin, or idarubicin, among others, and serve to eliminate the cancerous elements of the bone marrow, collaterally destroying healthy cells as well in order to allow for the marrow to repopulate with non-cancerous cells.[1] Given the harsh side effects of the chemotherapy and the intense course of treatment, elderly patients have more difficulty coping with the regimen. In addition to chemotherapy, bone marrow transplant may be required to encourage remission of the disease. The allogeneic stem cell transplantation is associated with procedural risk, particularly in elderly patients, as well as a risk of developing long-term complications such as chronic graft-versus-host disease (GVHD), which can prove to be fatal.[1] Given the severity of current treatments of AML and the poor prognosis for elderly patients, therapy for AML patients over the age of 65 often is solely palliative.

The Mixed-Lineage Leukemia Gene

The future of AML treatment must allow for a greater quality of life and prognosis than the current treatments used. Along with age, the other key prognostic factor in AML is chromosomal condition.[1] Many positive and negative genetic prognostic factors have been discovered in AML. The future of AML treatment lies in novel, personalized therapies specific to target each patient's unique genetic profile in the pathology of their disease. One gene often implicated in AML is the mixed-lineage leukemia (*MLL*) gene. Also known as *HRX* or *ALL1*, *MLL* encodes for a histone methyltransferase and lies within the breakpoint cluster region in the 11q23 chromosomal location and is involved in numerous translocations found in human

leukemias.[3] Dr. Patricia Ernst's analysis of WT *MLL* have shown multiple regions similar to the Trithorax gene in *Drosophila melanogaster*.[4] *MLL* positively regulates Hox genes, and loss-of-function analyses of *MLL* have shown *Hoxa4, a5, a7, a9, a10, c4, c5, c6, c9*[4], and *Meis1A*[5] to be direct downstream targets of *MLL*. *Hoxa9* and *Hoxa10* are significant players in hematopoietic processes as well as embryonic patterning. Hoxa9 has also been shown to be overexpressed in both human and murine AML[6], further emphasizing the importance of the *MLL* gene in AML leukemogenesis. Analysis of yolk sac and fetal liver by Yagi *et al*. after knocking out *Mll* showed a drastic reduction in hematopoietic cells, further implicating the importance of *Mll* in hematopoiesis.[14] *MLL* fusion mutations involve the translocation of the *MLL* gene with regions of other chromosomes. *MLL* fusion mutations have been shown to be sufficient to cause leukemia in a mouse model by both Lavau *et al*. using a myelomonocytic cell line and Corral *et al*. using an *Mll*-AF9 knock-in model of leukemogenesis.[7,8] In leukemias associated with a mutation of *MLL*, treatments that serve to correct the effects of the patient's specific mutation or gene therapy to correct the mutation itself could prove to be effective and less strenuous than the current chemotherapeutic regimens.

The Partial Tandem Duplication of *MLL*

In 1994, Caligiuri *et al*. used an *MLL* probe to study rearrangements in *MLL* in adults with *de novo* AML that do not have cytogenetically detectable translocations. It was found that cytogenetically undetectable rearrangements are associated with a poor prognosis.[9] The partial tandem duplication (PTD) of *MLL* is an in-frame duplication that does not create a truncated protein. *MLL*-PTD is a unique mutation as it maintains all functional domains. In our model, exons 5-11 are duplicated and inserted into intron 4. Exons 5-11 code for the the AT hooks, a

DNA-binding motif, and the methylbinding domain (Figure 1).[10] *MLL*-PTD was found by Schichman and Caligiuri to be present in approximately 5-10% of patients with AML, and is associated with a particularly poor prognosis.[9] Caligiuri *et al*. later characterized the mutation in AML with trisomy 11, again emphasizing the significance of *MLL*-PTD in human AML. Golub *et al*. determined that HOXA9, a target of *MLL,* was the gene whose expression correlates most with poor prognosis in human AML.[17] AML blasts with *MLL* fusions such as *MLL*-AF9 express the wild-type and fusion transcripts, and *MLL* fusions require the presence of the *MLL* wild-type gene to function.[21] Unlike *MLL*-AF9 fusions, Whitman *et al*. found that in *MLL*-PTD+ AML blasts, although the *MLL-WT* DNA is not lost, the *MLL*-WT transcript is for the most part absent, unlike the *MLL*-AF9 blasts.[12] These data suggested that the wild-type expression is repressed in patients with the *MLL* PTD, and this repression may result from DNA methylation of the *MLL* WT allele. Although the specific mechanisms by which *MLL*-PTD silences the *MLL* WT, and whether this has a causative role in leukemogenesis, are unknown,[11] the *MLL* PTD is a significant prognostic factor and therefore it is important to uncover the mechanisms that contribute to the biology of the leukemic cell to determine how to more effectively treat patients harboring the *MLL*-PTD mutation.

A Mouse Model of MLL-PTD

Our lab developed and studied a novel mouse model which expresses *Mll*-PTD under the control of the endogenous *Mll* promoter. The $Mll^{PTD/PTD}$ genotype proved to be embryonic lethal. Phenotypic analysis of the $Mll^{PTD/WT}$ mouse showed a high penetrance of axial skeletal defects, likely due to the elevated transcript levels of *Hoxa9*, as well as *Hoxa7* and *Hoxa10* as determined

by Dorrance *et al*.[13] Despite elevated transcript levels of these downstream Mll targets, *Mll*$^{PTD/WT}$ mice do not develop leukemia.

As aforementioned, Whitman *et al*. showed epigenetic silencing of *MLL*-WT in *MLL*-PTD+ AML patient blasts.[12] In order to discover the hematopoietic and leukemogenic mechanisms of the PTD mutation, it is thus important to look at how the PTD mutation functions (or doesn't function) in the absence of the Mll wild-type gene. Dorrance *et al*. crossed our lab's *Mll*$^{PTD/WT}$ mice with *Mll*$^{WT/-}$ mice obtained from the late Dr. Stanley Korsmeyer to obtain Mll$^{PTD/-}$ mice in order to study PTD alone, without *Mll*-WT. All F1 generation *Mll*$^{PTD/-}$ mice die by postpartum day 1.[11] Additionally, as Hanson *et al*. noted, Mll$^{-/-}$ mice are also nonviable, but die on average at embryonic day 10.5, as a result of lethal developmental abnormalities and abnormal apoptosis, further implicating the role of *Mll* in cell proliferation and development. The comparison of these results and the viability of *Mll*$^{PTD/-}$ embryos versus Mll$^{-/-}$ embryos shows that in absence of *Mll*-WT, *Mll*-PTD is able to partially compensate for embryonic development, even though it is still not enough to allow for sustained viability in these mice.[6] These findings are consistent with previous studies that show at least one copy of *Mll* is required for viability.[5, 14] However, because the PTD/- mice are not viable, these mice do not allow for the study of how the mutation alone affects hematopoiesis or leukemogenesis in adult mice (Figure 2).

Mll conditional knockout

In order to address the issue of embryonic lethality associated with PTD/- animals, we acquired *Mll* conditional knockout mice from the lab of Dr. Patricia Ernst. In these specific mice, *Mll* exons 3-4 are floxed, referred to as *Mll*-F (Figure 3a). *LoxP* flanked (floxed) regions of genes can be deleted in animals with Cre recombinase using *Mx1*-driven induction. This viral

process activates Cre recombinase to delete the floxed portion after induction with polyinosinic:polycytidylic acid (poly I:C). In these specific mice, *Mll* exons 3-4 are floxed, referred to as *Mll*-F (Figure 3a). Before induction, Mll-F mice are phenotypically identical to *Mll*-WT mice. However, after induction of *Mx1*-Cre recombinase, *Mll* exons 3-4 will be removed, resulting in deletion of the nuclear targeting sequence and subnuclear localization domains of *Mll*. Without these domains, *Mll* is no longer translocated to the nucleus and thus, no longer can perform nuclear function as a transcription factor., allowing for an effective knockout of the gene, referred to as *Mll*-ΔN[15] (Figure 3b).[15] Mice lacking Cre recombinase retain functional Mll-F in their genotype after induction and serve as controls.

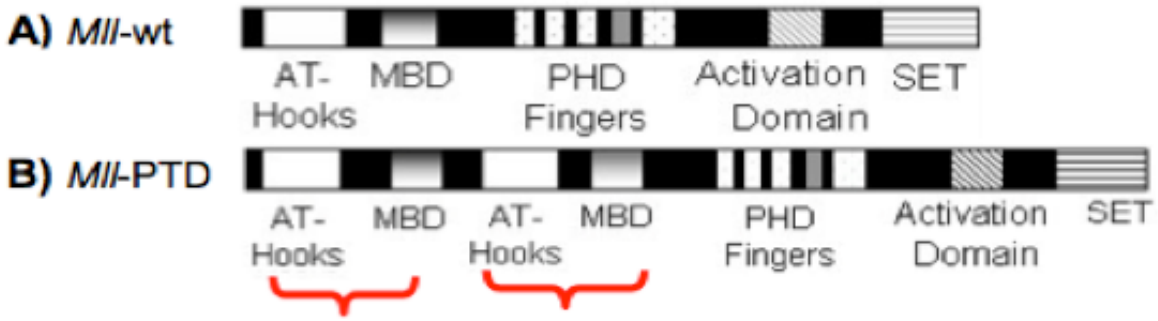
$Mll^{F/F}$ mice and $Mll^{WT/F}$ mice are viable and do not have any defects. Mice induced to obtain the $Mll^{WT/\Delta N}$ genotype show a reduction in bone marrow cellularity and hematopoietic function, but are capable of surviving with only one normal copy of *Mll*, as shown by Jude *et al*.[15] Adult $Mll^{F/F}$ mice that are induced to become $Mll^{\Delta N/\Delta N}$ mice die within 3 weeks of Cre recombinase induction due to bone marrow failure. These mice have no functional copies of *Mll* to maintain hematopoiesis, and thus hematopoietic stem cells and progenitors enter the cell cycle and are rapidly depleted.[15]

By crossing this conditional Mll deletion model with our *Mll*-PTD model, $Mll^{PTD/F}$ mice can be obtained. After induction of Mx1-Cre, these mice become $Mll^{PTD/\Delta N}$ mice, which allows for the study of the *Mll*-PTD mutation in the absence of any functional *Mll*-WT, comparable to human *MLL*-PTD+ AML blasts in which *MLL*-WT transcripts are absent. By studying hematopoietic (and potentially leukemogenic) processes in these mice, the specific role of the *MLL*-PTD mutation can be uncovered. It is possible that by further uncovering the phenotypes

and mechanisms associated with this mutation, therapeutic targets will be discovered and these findings can eventually be applied to treat *MLL*-PTD+ AML, despite its poor prognosis.

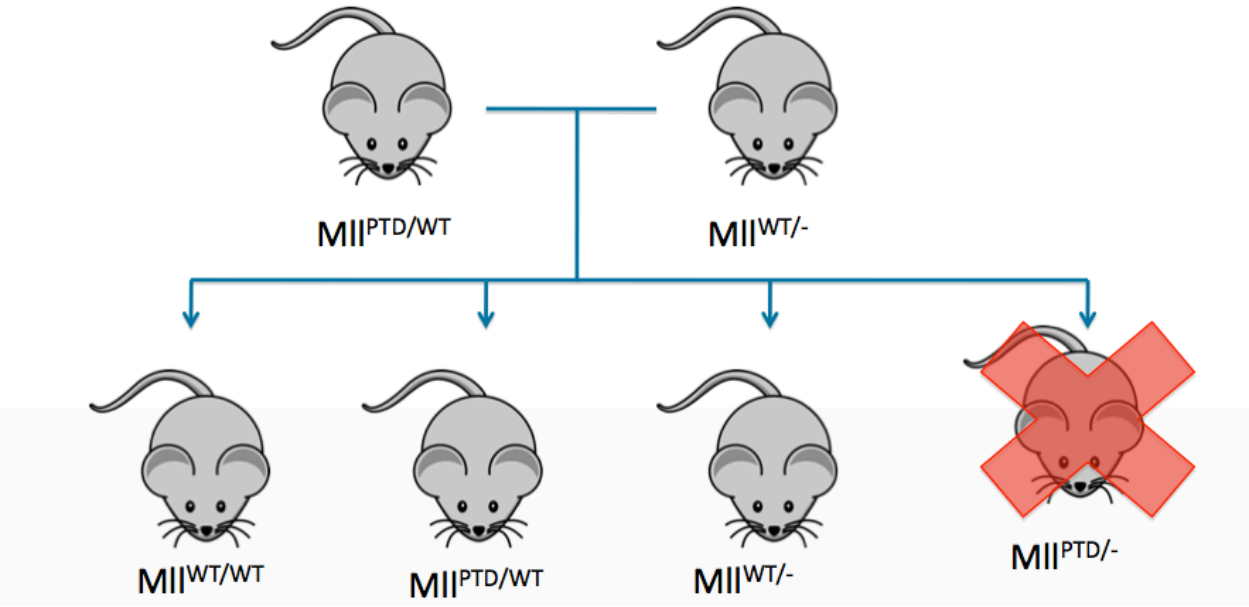
Introductory Figures:

Figure 1 – The Partial Tandem Duplication of *Mll*



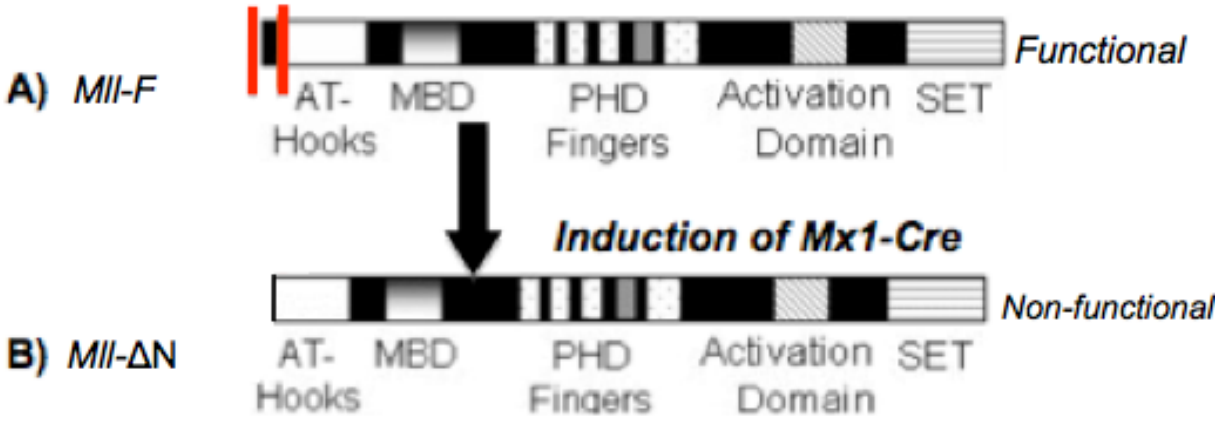
Mll-PTD maintains all functional domains, yet displays a tandem duplication of AT-hooks and the methylbinding domain.

Figure 2 – Breeding Model and Embryonic Lethality of $Mll^{PTD/-}$ mice



$Mll^{PTD/-}$ mice are not viable and die approximately at postpartum day 1. $Mll^{PTD/WT}$ mice are viable and do not develop leukemia.

Figure 3 – Cre Recombinase-driven Deletion of Subnuclear Localization Domains to turn *Mll*-F into *Mll*-ΔN



3a) *Mll*-F has *LoxP* flanked sites as denoted by the red lines. *Mll*-F is fully functional prior to Mx1-Cre induction. 3b) *Mll*-ΔN is formed from *Mll*-F mice containing Cre recombinase that are induced with poly I:C. *Mll*-ΔN is missing the subnuclear localization domain of *Mll*, and therefore is not translocated to the nucleus and serves as a null copy of nuclear *Mll WT*.

Materials and Methods:

Formation and observation of $Mll^{PTD/\Delta N}$ Mice

In order to generate mice expressing only *Mll*-PTD without *Mll*-WT, $Mll^{PTD/WT}$ mice were crossed with $Mll^{F/F}$ mice also expressing Mx1-Cre. Offspring from these breeders were genotyped using tail biopsies in order to test for presence of *Mll*-PTD, *Mll*-F, and Mx1-Cre recombinase. At six weeks of age, $Mll^{WT/WT}$, $Mll^{PTD/WT}$, $Mll^{F/F}$, $Mll^{WT/F}$, and $Mll^{PTD/F}$ mice were given 4 intraperitoneal injections of poly I:C on days 0, 2, 4, and 6. Poly I:C dosage = (10 x animal mass [g] + 50) µg poly I:C. Mice were weighed every day during the induction process, and every other day for 60 days following the induction. After induction, the resulting genotypes

of the aforementioned mice were $Mll^{WT/WT}$, $Mll^{PTD/WT}$, $Mll^{\Delta N/\Delta N}$, $Mll^{WT/\Delta N}$, and $Mll^{PTD/\Delta N}$. Mice lacking Cre recombinase do not experience a change in their genotype. Mice were then checked via PCR for complete deletion of *Mll*-F, and any mice showing residual expression of *Mll*-F were given an additional dose of poly I:C.

Examination, immunohistochemistry, and cytochemistry

Animals were observed daily for overt signs of sickness. Biweekly blood counts and differential assessment beginning at day 0 of induction were taken until animals displayed signs of bone marrow failure, as indicated by severe anemia, thrombocytopenia, or leucopenia, or until animals displayed extreme lethargy or >20% body weight loss. At this point, animals were sacrificed. Blood from the animals was used to make smears, followed by Wright-Giemsa staining. In addition, blood was saved in order to perform PCR to confirm deletion. Hematopoietic tissues, primarily bone marrow and spleen, were collected. These samples were used to perform immunophenotyping, to extract DNA to further confirm deletion via PCR, extract RNA, and prepare cell samples for microscopy and histological analysis.

Quantitative Real-time PCR

Quantitative RT-PCR was performed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) using a previously developed Taqman probe set for *Hoxa9* (Mm00439364_m1).

Statistical Analysis

The log-rank test was utilized to compare PTD/ΔN survival to the ΔN/ΔN control. A one-sample t-test was used to analyze RT-PCR data. All other results were analyzed using a one-way ANOVA test.

Genotyping and PCR

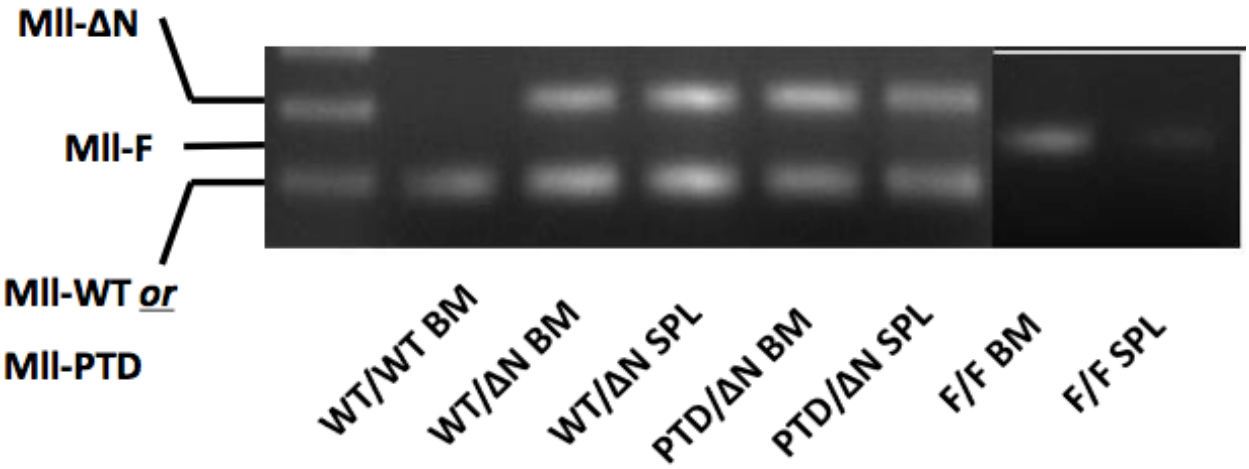
DNA was purified from tail samples as well as from bone marrow, spleen, and blood (Extract-N-Amp, Sigma). Standard PCR protocol was used to amplify the genes. The primers used are as follows:

Reaction	Forward Primer	Reverse Primer
Cre recombinase	CCTGTTTTGCACGTTCACCG	ATGCTTCTGTCCGTTTGCCG
Mll-F	CAGTGGACATTCCAACTCTTCAA	GATTGGCCAATGTCTCTCGTAGTAGGC
Mll-PTD	GAGCCTTGGCCCGAATGAAACTGT	CCGGCGAACGTGGCGAGAA

To test for deletion of *Mll*-F to *Mll*-ΔN, the above primers for *Mll*-F were used in conjunction with a third primer for deletion, which was CACCCAGCATTGCAGAGTCAG.

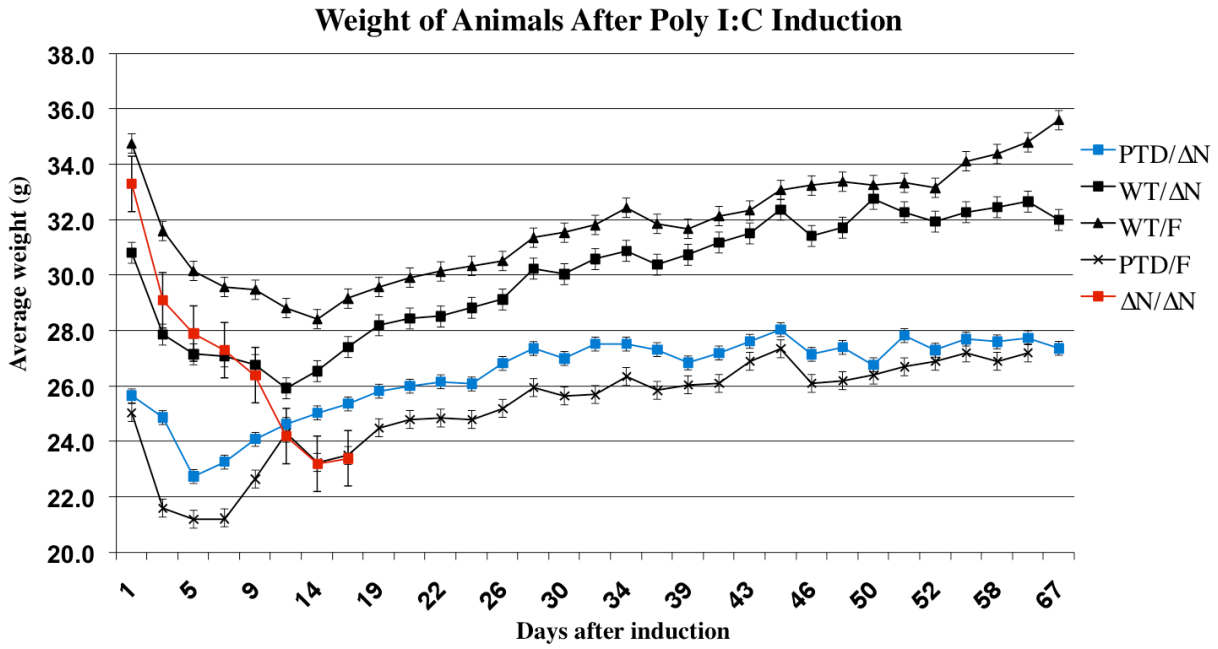
Results:

Figure 4 – Deletion of Mll-F in Induced Animals

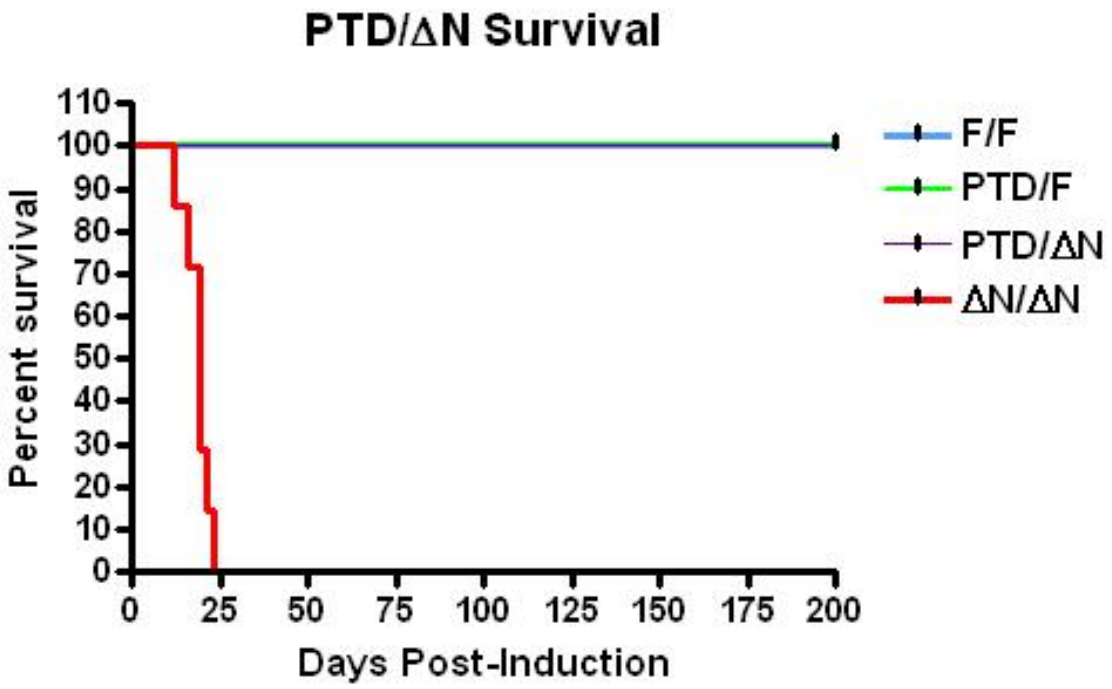


6. Mll-F is shown to be completely converted to *Mll*-ΔN in both bone marrow and spleen.

Figure 5 – The Result of poly I:C Induction

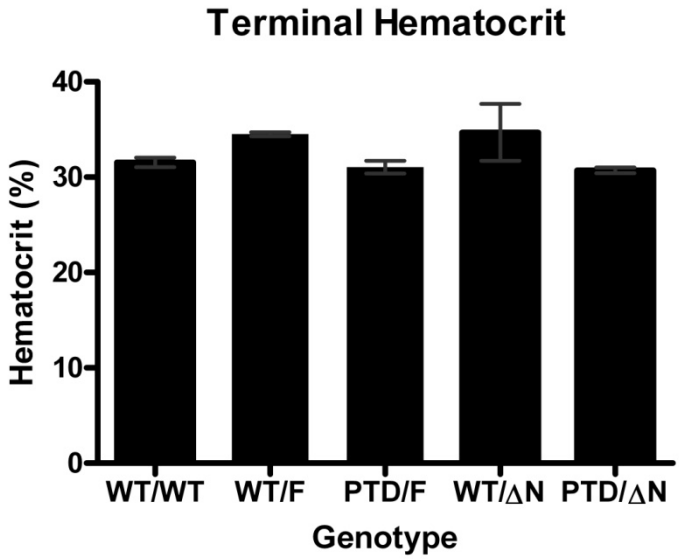


5a. All mice, regardless of genotype, show a marked drop in weight following the poly I:C induction. Following this weight drop, the mice regain and maintain their weight, excluding the ΔN/ΔN mice, which die due to bone marrow failure before their weight is stabilized. All mice with the PTD allele display stunted growth initially. Error bars represent standard deviation.

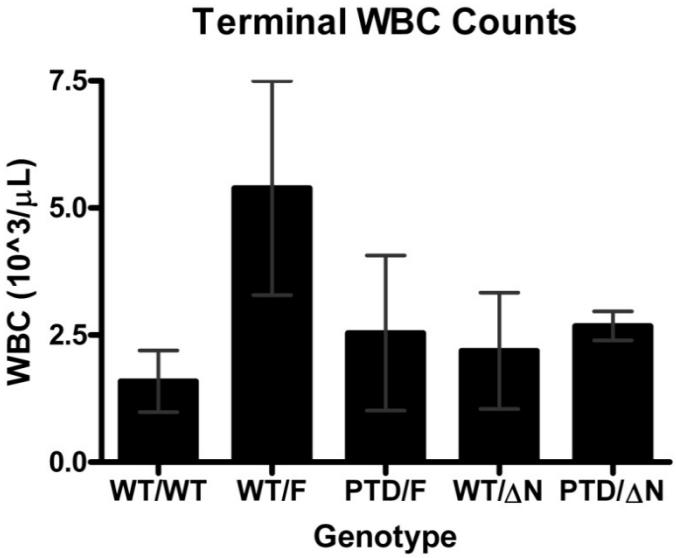


5b. Kaplan-Meier survival analysis shows that ΔN/ΔN mice die on average of 18 days after induction, and none survive past day 24 post-induction. Unlike ΔN/ΔN mice, PTD/ΔN mice do not succumb to bone marrow failure and do not die. Along with the age-matched control mice, PTD/ΔN mice have shown continued survival for over one year ($p<0.001$, $n=11$).

Figure 6 – Peripheral Blood Analysis upon Sacrifice

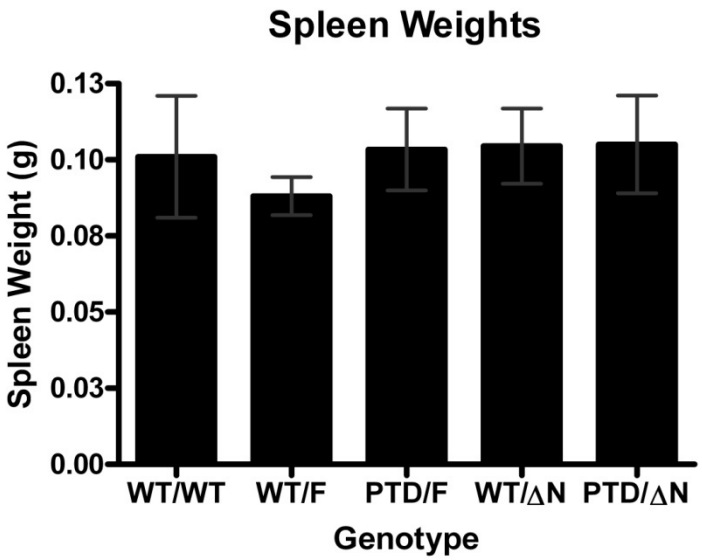


6a. PTD/ΔN mice maintain normal hematocrit levels that are consistent with other controls (n=3). The difference between PTD/ΔN hematocrit levels and age-matched control hematocrit levels is not statistically significant according to a one-way ANOVA test (p=0.0916). Error bars represent standard deviation.



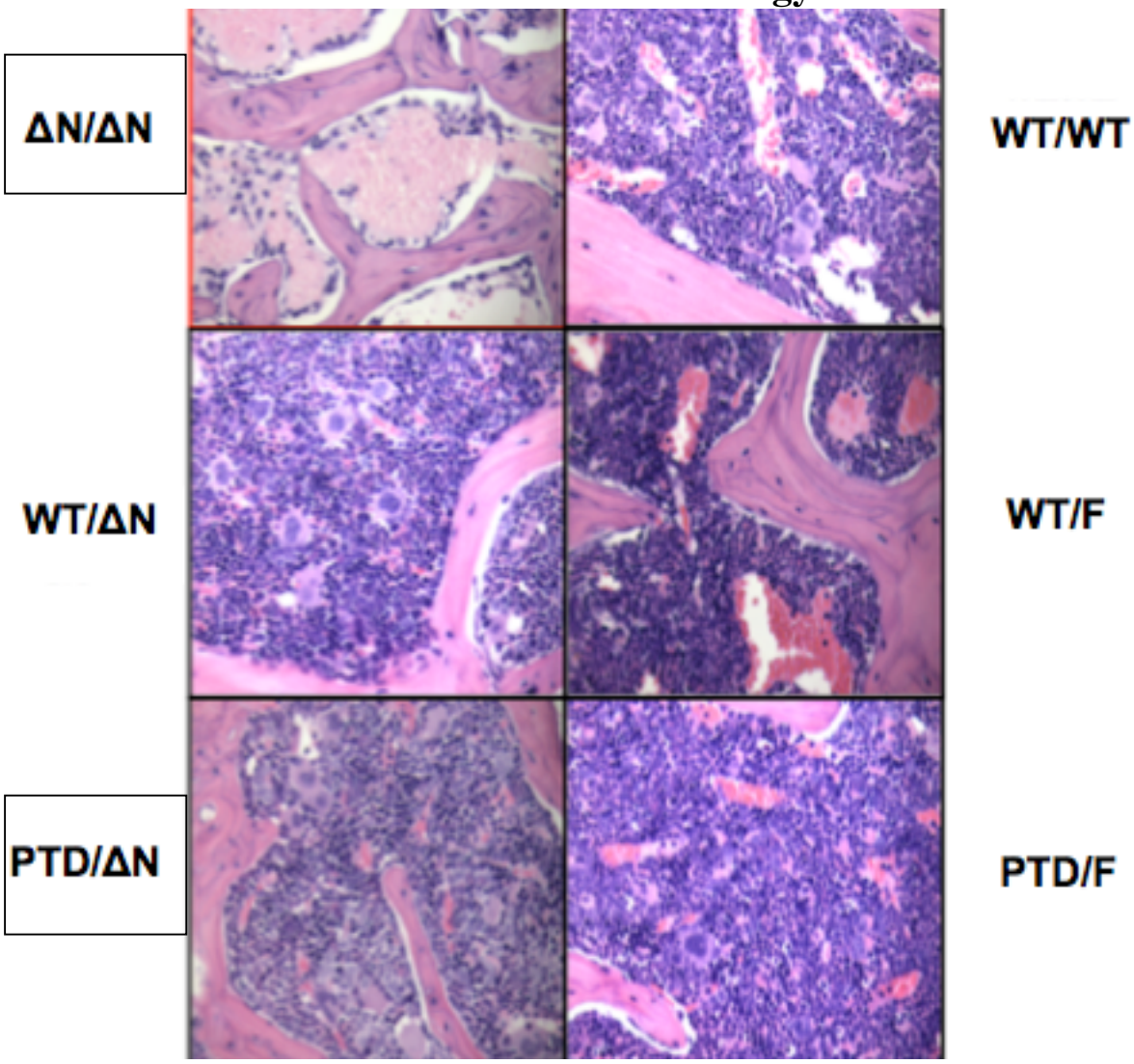
6b. PTD/ΔN mice also maintain normal white blood cell counts (n=3). Mice do not exhibit any signs of pre-leukemia as determined by WBC counts. The difference between PTD/ΔN WBC counts and age-matched control WBC counts is not statistically significant according to a one-way ANOVA test (p=0.1693). Error bars represent standard deviation.

Figure 7 – Histological Analysis of Hematopoietic Tissue



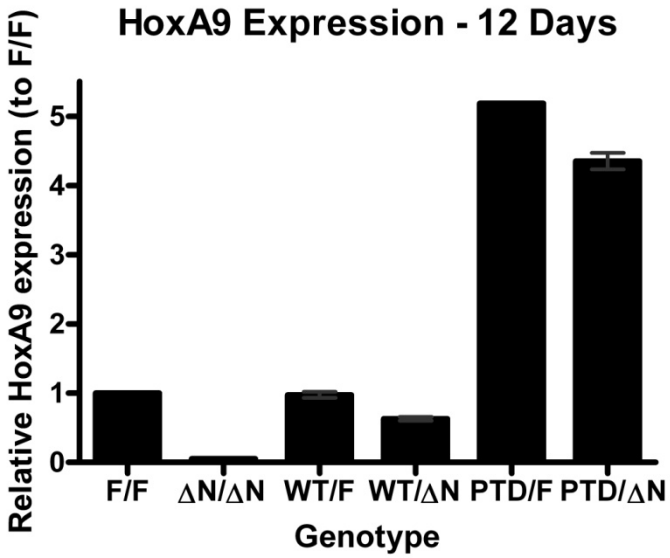
7a. PTD/ΔN mice do not have any statistically significant difference in spleen weight when compared to age-matched controls (p= 0.8463, n=4). Error bars represent standard deviation.

Bone Marrow Histology



7b. ΔN/ΔN mice show severe hypocellularity in the bone marrow upon sacrifice, indicative of bone marrow failure. Histological analysis of PTD/ΔN bone marrow shows no hypocellularity or other notable differences from age-matched controls. Slides were reviewed by a hematopathologist.

Figure 8 – Elevated transcript levels of downstream target of Mll in PTD mice



Mice with the PTD allele display elevated mRNA transcript levels of HoxA9 ($p=0.0225$) relative to age-matched F/F controls (n=3 for all samples except for PTD/F for which n=1). Error bars represent standard deviation.

Discussion

Based on the finding by Whitman *et al*. that *MLL*-WT is silenced in *MLL*-PTD+ AML blasts[12], this study sought to determine how the PTD mutation mediates hematopoiesis, if at all, in the absence of WT expression. Utilizing a novel mouse model, PTD was able to be expressed without the WT gene in PTD/ΔN mice, allowing for this study to circumvent the embryonic lethality of the expression of solely the *Mll*-PTD mutation. Leukemogenic *MLL* fusion mutations have been shown to require the presence of *MLL*-WT. Therefore, it was initially hypothesized that the *Mll*-PTD acts in a similar fashion and would not be able to carry out normal hematopoiesis without *Mll*-WT. Ultimately, the long-term goal of this study is to take

steps to positively impact *MLL*-PTD+ AML patients by uncovering mechanisms and therapeutic targets through an enhanced understanding of the basic biology of the disease.

Upon weighing both before and after poly I:C induction, it was noted that mice with the PTD allele weigh less than those without the PTD mutation. This is most likely attributed to defects in *Hox* gene expression[11,13], and this phenotypic difference did not affect the induction process or trends in weights of these mice. Upon induction, all mice displayed a drop in weight, characteristic of the immune system stimulation associated with the poly I:C[19]. The weights of the PTD/ΔN mice stabilized along the same time as the other age-matched controls (F/F, WT/F, and PTD/F). However ΔN/ΔN mice do not regain weight, and died 3 weeks after induction from bone marrow failure, a finding indicative of the absence of the *Mll*-F due to its conversion to *Mll*-ΔN (Figure 5a). Consistent with Dr. Ernst's previous findings, ΔN/ΔN mice succumb to bone marrow failure on average of 18 days as a result of the lack of any functional Mll to salvage hematopoesis. In contrast with the initial hypothesis, PTD/ΔN mice showed very different survival than the ΔN/ΔN mice ($p<0.001$). Indeed, no difference in survival time has been observed after over one year between the PTD/ΔN mice and the remaining age-matched controls, implying that the absence of *Mll*-WT does not lead to AML in PTD/ΔN mice. Toxicity of the poly I:C induction did not result in death of any PTD/ΔN mice (Figure 5b).

The ongoing survival of the PTD/ΔN mice could be attributed to three possible explanations. The first possibility was that the poly I:C induction was not complete and there were still some *Mll*-F alleles that were functioning in these mice and had not been converted to *Mll*-ΔN. Utilizing forward, reverse, and deleted reverse primers as previously indicated, PCR analysis of spleen, bone marrow, and blood displayed complete deletion of *Mll*-F, eliminating incomplete deletion as a feasible explanation (Figure 4). Another possibility is that the *Mll*-WT

is being shuttled into the nucleus by *Mll*-PTD via dimerization domains that may be retained. Although unlikely, this hypothesis is currently being tested by Dr. G. Huang at Cincinnati Children's. The third possibility was that our initial hypothesis was not correct, and *Mll*-PTD was indeed capable of effectively functioning and salvaging hematopoiesis in these mice. Blood counts of PTD/ΔN mice showed that hematocrit levels (Figure 6a) and white blood cell counts (Figure 6b) in these experimental mice are maintained at levels similar to those of the normal control mice. Leukemic and pre-leukemic mice often display enlarged spleen size, however PTD/ΔN mice, like the PTD/WT mice, (Figure 7a) did not have enlarged spleens. Furthermore, histological analysis of bone marrow displayed considerable hypocellularity in the ΔN/ΔN mice, indicative of depletion of hematopoietic stem/progenitors, resulting in bone marrow failure. However, along with the age-matched control mice, PTD/ΔN mice displayed no hypocellularity and no signs of pre-leukemia in the bone marrow (Figure 7b). All of these results lead to the conclusion that the Mll-PTD protein is in fact capable of salvaging hematopoesis in adult mice lacking *Mll*-WT expression.

Analysis of all major adult hematopoietic tissues indicate that PTD/ΔN mice are capable of maintaining hematopoiesis at levels relative to the wild-type controls. Thus, it follows that the PTD mutation, contrary to leukemogenic MLL fusions, is capable of salvaging hematopoiesis at normal levels in the absence of *Mll*-WT. Going on 500 days after induction, PTD/ΔN mice show no signs of leukemia and continue to live and reproduce normally. Although no pre-leukemia phenotypic signs are found in these mice, preliminary data showed that transcript levels of *Hoxa9*, a major downstream target of Mll, were elevated in both PTD/F and PTD/ΔN mice (Figure 8), suggesting that *Mll*-PTD is acting in a gain-of-function manner, confirming the findings of Dorrance *et al*[11] and Whitman *et al*[12] in this experiment's adult mice lacking *Mll*-WT

expression. The elevated mRNA transcript levels do not lead to leukemogenesis or altered hematopoiesis, but multiple possibilities exist to explain the implications of *Mll*-PTD acting in a gain-of-function manner. Firstly, transcript levels are not always indicative of translated protein levels, and it is possible that the elevated *HoxA9* transcript levels shown in PTD mice do not translate to elevated HoxA9 protein expression. It follows that one such future experiment to be conducted is to utilize western blotting to observe HoxA9 protein levels in the PTD/ΔN mice. Alternatively, the elevated transcript levels could simply be the first "hit" to leukemogenesis, and the accumulation of other mutations would combine either additively or synergistically to lead to leukemia. Recently our lab published on a double knock-in model of *Mll*-PTD together with the *Flt3*-ITD, the internal tandem duplication mutation of Fms-like tyrosine kinase 3. It was shown that AML did not develop in the knock-in mice with only the PTD or only ITD mutation, although some models have shown that certain retrovirally-expressed *Flt3*-ITD mutations can be leukemogenic.[20] Leukemia does develop in mice with both the PTD and the ITD, supporting the hypothesis that the gain-of-function of Mll PTD is necessary but insufficient to cause AML and thus requires a second mutation, like Flt3-ITD.[20]

This project has uncovered a gain-of-function role for the *Mll*-PTD mutation in adult hematopoiesis. Armed with the basic biology of the functioning of *Mll*-PTD, we hope to eventually develop targeted therapies for patients with *MLL*-PTD+ AML. In order to reach that point, we must still further uncover the mechanisms by which the *MLL*-PTD mutation causes leukemogenesis. One future experiment to further investigate into the role of *MLL*-PTD is to perform serial competitive repopulation studies in order to determine the self-renewal capacity of PTD/ΔN hematopoietic stem cells and progenitor cells relative to controls. Given the gain-of-function action of *Mll*-PTD, I hypothesize that these studies will show an increased self-renewal

capacity and repopulation capability of cells with the PTD mutation, a finding that would further implicate the gene as a contributing factor in leukemogenesis. In addition to this, continued monitoring of the PTD/ΔN mice is important. As aforementioned, age and chromosomal condition are the two key factors in leukemogenesis and prognosis of AML. Although our mice are currently entering into old age, it is still possible that age could affect progression of the disease, if any arises.

Acknowledgements:

I would firstly like to thank my project advisor, Dr. Michael Caligiuri, for all of his guidance, insight, and instruction. Without his instruction, I would be neither the researcher nor the person that I am today. I would also like to thank Nicholas Zorko, who graciously took me under his wing and has taught me everything that I know. Without him this work would not be possible.

Next, I would like to thank Dr. Adrienne Dorrance and Dr. Susan Whitman for their assistance in the completion of this thesis, as well as their guidance in the execution of experiments. I would like to thank Dr. Kelsie Bernot, Gabriele Marcucci, Ronald Siebenaler, Kathleen McConnell, Elshafa Ahmed, Charlene Mao, Anjali Mishra, and all other members of Caligiuri Lab for their hard work and dedication. I would also like to thank Dr. Caroline Breitenberger and Dr. Noel Paul for serving on my thesis committee. This work was supported by the Pelotonia Undergraduate Research Fellowship. Any opinions, findings, and conclusions expressed in this material are those of the author and do not necessarily reflect those of the Pelotonia Fellowship Program.

Finally, I would like to thank Dr. Burhan Yanes, Dr. Giti Rostami, and Arianna Yanes for their expert advice and support.

Citations:

1. Stone, R. M., O'Donnell, M. R., & Sekeres, M. A. (2004). Acute myeloid leukemia. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*, *2004*(1), 98-117. doi:10.1182/asheducation-2004.1.98

2. Vardiman, J. W., Thiele, J., Arber, D. A., Brunning, R. D., Borowitz, M. J., Porwit, A., Harris, N. L., et al. (2009). The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*, *114*(5), 937-51.

3. Ziemin-van der Poel, S., McCabe, N. R., Gill, H. J., Espinosa, R., III, Patel, Y., Harden, A., Rubinelli, P., Smith, S. D., LeBeau, M. M., Rowley, J. D., and et al. (1991). Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias [published erratum appears in Proc Natl Acad Sci U S A 1992 May 1;89(9):4220]. Proc Natl Acad Sci USA *88,* 10735-10739.

4. Ernst P, Wang J, Korsmeyer SJ. The role of MLL in hematopoiesis and leukemia. Curr Opin Hematol. 2002;9(4):282–287.

5. Milne, T. A., Dou, Y., Martin, M. E., Brock, H. W., Roeder, R. G., & Hess, J. L. (2005). MLL associates specifically with a subset of transcriptionally active target genes. Proc Natl Acad Sci USA, *102*(41), 14765-70.

6. Hanson, R. D., Hess, J. L., Yu, B. D., Ernst, P., van Lohuizen, M., Berns, A., van der Lugt, N. M., et al. (1999). Mammalian Trithorax and polycomb-group homologues are antagonistic regulators of homeotic development. Proc Natl Acad Sci USA, *96*(25), 14372-7.

7. Lavau, C., Szilvassy, S. J., Slany, R., & Cleary, M. L. (1997). Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. *The EMBO journal*, *16*(14), 4226-37.

8. Corral, J., Lavenir, I., Impey, H., Warren, A. J., Forster, A., Larson, T. A., Bell, S., et al. (1996). An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. *Cell*, *85*(6), 853-61.

9. Caligiuri, M. A., Schichman, S. A., Strout, M. P., Mrzek, K., Baer, M. R., Frankel, S. R., Barcos, M., et al. (1994). Molecular Rearrangement of the ALL-1 Gene in Acute Myeloid Leukemia without Cytogenetic Evidence of 11q23 Chromosomal Translocations, 370-373.

10. Schichman, S. A. (1994). ALL-1 Partial Duplication in Acute Leukemia. *Proceedings of the National Academy of Sciences*, *91*(13), 6236-6239.

11. Dorrance, A. M., Liu, S., Chong, A., Pulley, B., Nemer, D., Guimond, M., Yuan, W., et al. (2008). The Mll partial tandem duplication: differential, tissue-specific activity in the presence or absence of the wild-type allele. *Blood*, *112*(6), 2508-11.

12. Whitman, S. P., Liu, S., Vukosavljevic, T., Rush, L. J., Yu, L., Liu, C., Klisovic, M. I., et al. (2005). The MLL partial tandem duplication: evidence for recessive gain-of-function in acute myeloid leukemia identifies a novel patient subgroup for molecular-targeted therapy. *Blood*, *106*(1), 345-52.

13. Dorrance, A. M., Liu, S., Yuan, W., Becknell, B., Arnoczky, K. J., Guimond, M., Strout, M. P., Feng, L., Nakamura, T., Yu, L., Rush, L. J., Weinstein, M., Leone, G., Wu, L., Ferketich, A., Whitman, S. P., Marcucci, G., and Caligiuri, M. A. (2006). Mll partial tandem duplication induces aberrant Hox expression in vivo via specific epigenetic alterations. Journal of Clinical Investigation 116, 2707-2716.

14. Yagi, H., Deguchi, K., Aono, A., Tani, Y., Kishimoto, T., and Komori, T. (1998). Growth disturbance in fetal liver hematopoiesis of Mll-mutant mice. Blood *92,* 108-117.

15. Jude CD, Climer L, Xu D, Artinger E, Fisher JK, and P Ernst. Unique and independent roles for MLL in adult hematopoietic stem cells and progenitors. Cell Stem Cell 1(3);doi:10.1016/ j.stem.2007.05.019, 2007

16. Cimino, G., Moir, D., Cananni, O., Williams, K., Crist, W., Katzav, S., Cannizzaro, L., Lange, B., Nowell, P., Croce, C., and Canaani, E. (1991). Cloning of *ALL-1,* the locus involved in leukemias with the t(4;11)(q21;q23), t(9;11)(p22;q23), and t(11;19)(q23;p13) chromosome translocations. Cancer Res *51,* 6712-6714.

17. Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., Bloomfield, C. D., and Lander, E. S. (1999). Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science *286,* 531-537.

18. Gu, Y., Nakamura, T., Alder, H., Prasad, R., Canaani, O., Cimino, G., Croce, C. M., and Canaani, E. (1992). The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. Cell *71,* 701-708.

19. *Poly(I:C) Vaccigrade: Vaccine Adjuvant TRL3 Agonist*. N.p., n.d. Web. 24 June 2012. <http://www.invivogen.com/polyic-vaccigrade>.

20. Zorko, N. A., Bernot, K. M., Whitman, S. P., Siebenaler, R. F., Ahmed, E. H., Marcucci, G. G., **Yanes, D. A.,** McConnell, K. K., Mao, C., Kalu, C., Zhang, X., Jarjoura, D., Dorrance, A. M., Heerema, N. A., Lee, B.H., Huang, G., Marcucci, G., Caligiuri, M. A. (2012). Mll-

partial tandem duplication and Flt3-internal tandem duplication in a double knock-in mouse recapitulates features of counterpart human acute myeloid leukemias. *Blood*.

21. Thiel, A.T., Blessington, P., Zou, T., Feather, D., Wu, X., Yan, J., Zhang, H., Liu, Z., Ernst, P., Koretzky, G.A., *et al*. (2010). MLL-AF9-Induced Leukemogenesis Requires Coexpression of the Wild-Type *Mll* Allele. Cancer Cell *17(2,)* 148 – 159.